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(54) Title: IMPROVED BIOSENSOR

(57) Abstract

The present invention provides a membrane based biosensor. The biosensor includes an electrode and a passivating layer bound to the electrode. A lipid membrane incorporating ionophores, the conductivity of the membrane being dependent on the presence or absence of an analyte, is bound to the passivating layer in a manner such that an ionic reservoir exists between the membrane and the passivating layer. Reservoir spanning molecules spanning the ionic reservoir are also included. These molecules are covalently attached at one end to the membrane and at the other to the passivating layer. The incorporation of this passivating provides greater stability to the sensor.

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Improved Biosensor

The present invention relates to an improved membrane based biosensor and to methods of improving the stability of membrane based biosensors.

Biosensors based on ion channels or ionophores contained within lipid membranes that are deposited onto conducting electrodes, where the ionophores are switched in the presence of analyte molecules have been described in International Patent Application Nos PCT/AU88/00273; PCT/AU89/00352; PCT/AU90/00025; and PCT/AU93/00509 (the disclosures of which are incorporated herein by way of reference). As these biosensors rely on changes in ion conduction through the membrane, usually mediated by an ionophore, it is important that there exists an ionic reservoir between the electrode and the lipid membrane. It is also important that the lipid membrane is at least in part linked to the reservoir and that the membrane is at least in part tethered to the conductive substrate. The stability of the substrate and the stability of the link between the reservoir and the substrate will influence the stability of the total membrane plus ionic reservoir system and thus the stability of the biosensor membrane.

The present inventors have now found that the stability of the biosensor membrane can be improved by incorporating a passivating layer between the reservoir region and the conducting substrate. This passivating layer serves to increase the bonding between the reservoir forming linkers and the conductive substrate as well as serving to protect the conductive substrate surface from corrosive or electrochemical effects of the aqueous solution. This would improve the stability of the biosensor both during storage and during the actual measurement of the biosensor response on addition of analyte containing sample. A reduction in the drift of the biosensor response during analyte addition is also obtained.

Generally, formation of the reservoir functionality onto conductive substrates is through binding of individual reservoir molecules with the substrate. The present inventors have determined that by introducing binding, passivating layer between the reservoir forming molecules and the electrode the stability of the whole membrane system is improved. There is also preferably binding between the molecules making up the passivating

layer. This binding may include van der Waal's forces, hydrogen bonding forces, ionic interactions or covalent linkage. Furthermore, if a thin, passivating layer is formed between the reservoir and the conductive surface such that water and ions are restricted from interacting with the conductive substrate then corrosive effects such as electrochemical degradation are minimised and the stability of the membrane is improved. This thin passivating layer may be eletrically insulating in nature. The ionic reservoir, in this case, has a reduced contact with the conductive substrate directly and information about the ion flux into or out of the ionic reservoir can be obtained by a variety of electronic transient pulse techniques commonly used to measure the charging and discharging of resistor/capacitor circuits.

Additionally, by introducing this thin layer between the ionic reservoir and the conductive substrate, then the spacing and orientation of the ionic reservoir forming molecules is determined by the structure of the thin layer substance rather than by the surface crystal orientation of the underlying conductive substrate.

Accordingly in a first aspect the present invention consists in a membrane based biosensor, the biosensor including an electrode, a passivating layer bound to the electrode, a lipid membrane incorporating ionophores, the conductivity of the membrane being dependent on the presence or absence of an analyte, an ionic reservoir between the membrane and the passivating layer, and spanning molecules spanning the ionic reservoir the molecules being covalently attached at one end to the membrane and at the other to the passivating layer.

In a preferred embodiment the biosensor includes a plurality molecules of following general structure:

A-B-C-D

in which:

A is a hydrophobic group of between 2-50 methylene units in length;

B is a group which spans the ionic reservoir;

C is a group capable of hydrogen bonding, forming van der Waal's interactions, ionic bonding or covalent bonding with other molecules contained within the passivating layer; and

D is a group which binds to the conducting substrate.

In a preferred embodiment A is a hydrocarbon group of between 2-50 methylene units long, a phytanyl group, an unsaturated hydrocarbon of between 2-50 methylene groups long, a membrane spanning lipid, an archaebacterial lipid, a lipid hydrocarbon group, or a gramicidin derivative. It is presently preferred that A is a hydrocarbon group or an unsaturated hydrocarbon group of between 8-26 methylene units long or a gramicidin derivative.

In another preferred embodiment B is an oligoethylene glycol of between 4 to 20 ethylene glycol units long. Alteratively B may be repeating subunits of oligoethylene glycol of between 2 and 6 ethylene glycol units, the subunits being linked together via ester, amide or other linkages. It is highly preferred that in this arrangement that the linkages do not promote hydrogen bonding between the groups spanning the reservoir. In this regard it is preferred that the linkages are tertiary amides. As will be appreciated it is also highly preferred that linkages resistant to hydolysis such as tertiary amides are used.

In a further preferred embodiment group C of the above embodiment includes a secondary amide capable of hydrogen bonding with other amides; a hydrocarbon group capable of forming van der Waals interactions with other hydrocarbon groups, or a polymerisable group.

In a still further preferred embodiment D is a thiol, disulfide, sulfide, phosphine, silane or carboxylate.

It is further preferred that the group C in the above embodiment consists in a saturated hydrocarbon group of between 2 to 50 methylene units long, more preferably 8 to 30 methylene units long.

In a preferred embodiment the biosensor includes a plurality molecules of following general structure:

C-D

in which C and D are as defined above.

As will be recognised in the biosensor of the present invention group A will be within and form part of the membrane, group B will span the ionic reservoir and groups C and D will be within and form part of the passivating layer.

In a preferred embodiment the biosensor has a structure as shown in Figure 1.

In a further preferred embodiment the passivating layer has reduced permeability towards water and towards ions thus protecting the surface of the conductive substrate from destabilising effects due to water or ions.

In a preferred embodiment the biosensor includes a plurality molecules of following general structure:

A-B-C-D

in which:

A is a hydrophobic group such as a hydrocarbon group of between 2-50 methylene units long, a phytanyl group, an unsaturated hydrocarbon of between 2-50 methylene groups long, a membrane spanning lipid or archaebacterial lipid analog, a lipid hydrocarbon group, or a gramicidin derivative;

B is a group which spans the ionic reservoir;

C is a group that inhibits the permeation of water or ions to the conductive surface; and

D is a group capable of being attached to a conducting substrate such a a thiol, disulfide, sulfide, phosphine, silane, carboxylate or other group known to form strong interactions with surfaces.

In a preferred embodiment A is a hydrocarbon group or an unsaturated hydrocarbon group of between 8-26 methylene units long.

In another preferred embodiment B is an oligoethylene glycol of between 4 to 20 ethylene glycol units long. Alteratively B may be repeating subunits of oligoethylene glycol of between 2 and 6 ethylene glycol units, the subunits being linked together via ester, amide or other linkages. It is

highly preferred that in this arrangement that the linkages do not promote hydrogen bonding between the groups spanning the reservoir. In this regard it is preferred that the linkages are tertiary amides. As will be appreciated it is also highly preferred that linkages resistant to hydolysis such as tertiary amides are used.

In a further preferred embodiment group C includes a hydrocarbon group capable of forming van der Waals interactions with other hydrocarbon groups. The hydrocarbon group may contain a polymerisable group. Additionally the group may be functionalised at the distal end to the group D in order to modulate the function of the ion reservoir.

It is further preferred that the group C in the above embodiment comprises a saturated hydrocarbon group of between 2 to 50 methylene units long, more preferably between 8 to 30 methylene units long.

In a preferred embodiment the biosensor includes a plurality molecules of following general structure:

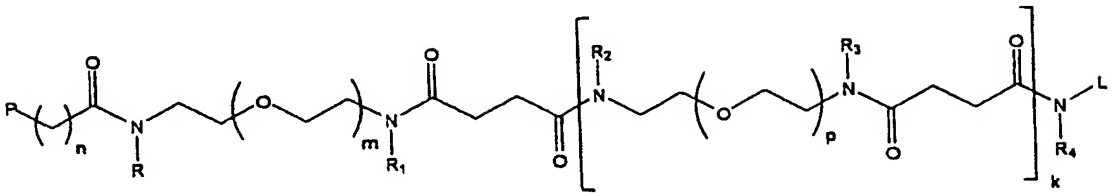
C-D

in which C and D are as defined above.

In a yet another preferred embodiment the passivating layer is strongly bound to the conductive substrate and the functionality that make up the ionic reservoir.

Additionally it is preferred that the group C may be functionalised at the distal end to the group D. This modification may include the incorporation of hydrophilic or hydrophobic groups, amides, alcohols, acids, amines, acrylamide or acrylate or other polymerisable groups that may be used to further stabilise the passivating layer and may be used to modulate the ion reservoir function.

It is further preferred to use molecules such as those shown below and in figures 2 and 3.



where:
n = 8 to 16
m = 1 to 10
p = 1 to 10
k = 0 to 10
R, R_1, R_2, R_3, R_4 = independently H, methyl, ethyl
L = hydrocarbon such as a phytanyl chain, or other lipidic hydrocarbon
P = a thiol, disulfide, sulfide, or other group for attaching to metal surfaces such as gold, platinum, palladium, silver etc; or a silane or alkoxy silane or chloro silane for attaching to silica or metal oxide or other conductive surface as is known to those skilled in the art.

In a second aspect the present invention consists in a biosensor of any aspects one or two wherein the flux of ions mediated by an ionophore through the membrane into the ionic reservoir is measured by transient electrical pulse techniques.

In order that the nature of the present invention may now be more clearly understood preferred forms thereof will now be described with reference to following non-limiting examples and Figures, in which:

Figure 1 is a schematic representation of part of the biosensor of the present invention;

Figures 2 and 3 show structures of preferred compounds used in the present invention;

Figure 4 shows the structure of DLP;

Figure 5 shows the structures of DLP-C11 and DLP-C16:

Figure 6 shows the structures of MSLOH and MSL-4XB;
Figure 7 shows the structure of gramicidin (gA);
Figure 8 shows the structure of gA-YY; and
Figure 9 shows the structure of gA-5XB

MATERIALS AND METHODS

ABBREVIATIONS

DLP	'Double length' phytanol, see Figure 4
PC lipid mixture	A mixture of 1,2-di(3RS,7R,11R-phytanyl)-glycero-3-phosphocholine and 1,2-di(3RS,7R,11R-phytanyl)glycerol in a 7:3 ratio
DLP-C11	'Double length' phytanol, attached to C11 foot, see Figure 5
DLP-C16	'Double length' phytanol, attached to C16 foot, see Figure 5
BnDS	Benzyl disulfide
MSLOH	'Membrane spanning lipid', see Figure 6
MAAD	Mercaptoacetic acid disulfide
MSL-4XB	Biotinylated membrane spanning lipid, see Figure 6
gA	Gramicidin, see Figure 7
gA-YY	Tethered gramicidin, see Figure 8
DPEPC	1,2-di(3RS,7R,11R-phytanyl)-glycero-3-phosphocholine
GDPE	1,2-di(3RS,7R,11R-phytanyl)glycerol
gA-5XB	Biotinylated gramicidin, see Figure 9
DCC	Dicyclohexylcarbodiimide
DMAP	Dimethylaminopyridine

11-Mercaptoundecanoic acid (G)

11-Bromoundecanoic acid (20.0 g, 0.075 mol) and thiourea (6.3 g, 1.1 equiv.) were refluxed in ethanol (250 mL) overnight. Sodium hydroxide (6.3 g) in water (20 mL) was added and the mixture was further refluxed for 2 hr. The solution was cooled and acidified with 1N HCl. Ethanol was evaporated under reduced pressure and the aqueous residue was extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$) and evaporated to give white powder. Yield: 15.0 g (91%)
^1H $\delta(CDCl_3)$: 1.32 (m, 13H), 1.62 (m, 4H), 2.35 (t, 2H), 2.52 (m, 2H).

Undecanoic acid disulphide (D)

11-Mercaptoundecanoic acid **(G)** (3.0 g, 13.7 mmol) and triethylamine (4 mL, 2.1 equiv.) were dissolved in $CH_2Cl_2/CH_3OH(1:1)$ mixture (20 mL) and cooled. A solution of iodine in methanol was added until excess I_2 was present. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2. The solution was acidified using 3N HCl and little methanol was added to dissolve insoluble solid. The organic phase was separated, dried ($MgSO_4$) and evaporated to yield a solid residue. The product was recrystallised once each from acetone and toluene. Yield: 1.2 g (40%)

^1H $\delta(CDCl_3)$: 1.26 (br, 24H), 1.62 (br, 8H), 2.37 (t, 4H), 2.65 (m, 4H).

11-Mercaptoundecanol (H)

11-Mercaptoundecanoic acid (G) (1.0 g, 4.6 mmol) was dissolved in dry THF (20 mL) and borane dimethyl sulphide (3 mL, 1.1 equiv.) was slowly added under N_2 atmosphere. The mixture was stirred at room temperature for 4 hr. Water was added to the solution was extracted using ethyl acetate. The organic extract was washed with 1N HCl, water and saturated sodium bicarbonate solution successively. After drying ($MgSO_4$), the solvent was evaporated to give a clear oil. The crude oil was purified by column chromatography [eluant: hexane/ethyl acetate (8:2)]. Yield: 0.45 g (48%)

^1H $\delta(CDCl_3)$: 1.28 (br, 18H), 1.58 (br, 4H), 2.52 (q, 2H), 3.65 (q, 2H).

Undecanol disulphide (E)

11-undecanoic acid disulphide **(D)** (0.5 g, 4.6 mmol) was dissolved in dry THF (20 mL) and borane dimethyl sulphide (3 mL, 1.1 equiv.) was slowly added under N_2 atmosphere. The mixture was stirred at room temperature for 5 hr. Water was added to the solution was extracted using ethyl acetate. The organic extract was washed with 1N HCl, water and saturated sodium bicarbonate solution successively. After drying ($MgSO_4$), the solvent was evaporated to give a white powder. Yield: 0.15 g (32%)

^1H $\delta(CDCl_3)$: 1.28 (br, 28H), 1.58 (br, 8H), 2.68 (t, 4H), 3.63 (t, 4H).

Compound (J)

Undecanoic acid disulphide **(D)** (0.43, 1.0 mmol) and succinic anhydride (0.23 g, 2.0 mmol) were dissolved in CH_2Cl_2 (30 mL). DMAP (20 mg, 0.1 equiv.) and DCC (0.41, 2.0 mmol) were added and the mixture was heated at 50 °C for 1 hr. The mixture was filtered and the filtrated was added with ethanolamine (1.5 equiv.). After stirring for further 2 hr. insoluble powder was collected and was purified by column chromatography [eluant: methanol/dichloromethane (1:9)]. Yield: 0.18 g (35%)
^1H $\delta(CDCl_3)$: 1.38 (br, 24H), 1.68 (br, 8H), 2.22 (t, 4H), 2.71 (t, 4H), 3.31 (t, 4H), 3.62 (t, 4H).

63 mg (0.12 mmol) of the purified disulphide was dissolved in a ethanol/water mixture (20:3) (23mL). Triphenylphosphine (47 mg, 1.5 equiv.) was added and the mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure and the solid residue was purified by column chromatography [eluant: methanol/CH_2Cl_2 (8:92)]. Yield: 10 mg (16%)
^1H $\delta(CDCl_3)$: 1.35 (br, 12H), 1.62 (br, 4H), 2.22 (t, 2H), 2.52 (t, 2H), 3.32 (t, 2H), 3.62 (t, 2H).

Phytanol (1)

Phytol (5.0 g, 16.7 mmol) was stirred in ethanol (50 mL) in the presence of Raney nickel (1.0 g, 50% slurry in water) overnight under H_2 atmosphere. The catalyst was filtered off and the solvent evaporated to give a clear oil. Yield: 4.9 g (97%)
^1H $\delta(CDCl_3)$: 0.87 (m, 15H), 1.25 (br, 24H), 3.70 (br, 2H).

Phytanyl succinate (2)

Phytanol (1) (5.0 g, 16.7 mmol) and succinic anhydride (2.5 g, 1.5 equiv.) were dissolved in pyridine (20 mL) and stirred at room temperature overnight. Pyridine was removed under reduced pressure and 0.5N HCl (100 mL) was added. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$) and evaporated to give an oil.

The crude product was purified by column chromatography [eluant: ethyl acetate/hexane (15:85)]. Yield: 5.5 g (82%)
^1H δ($CDCl_3$) : 0.87 (m, 15H), 1.25 (br, 24H), 2.65 (m, 4H), 4.18 (t, 2H).

Compound (3)

Phytanyl succinate **(2)** (4.2 g, 0.01 mol), tetraethylene glycol (10.2 g, 5 equiv.) and 4-dimethylaminopyridine(DMAP) (0.13 g, 0.1 equiv.) were dissolved in CH_2Cl_2 (50 mL). 1,3-Dicyclohexylcarbodiimide(DCC) (4.35 g, 2 equiv.) was added and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by column chromatography [eluant: ethyl acetate/hexane (6:4)]. Yield: 2.6 g (43%)
^1H δ($CDCl_3$) : 0.85 (m, 15H), 1.25 (br, 24H), 2.65 (t, 4H), 3.67 (m, 14H), 4.12 (t, 2H), 4.27 (t, 2H).

Compound (4)

Compound **(3)** (1.42 g, 2.5 mmol) and succinic anhydride (0.37 g, 1.5 equiv.) were dissolved in pyridine (20 mL) and stirred at room temperature overnight. Pyridine was removed under reduced pressure and 1N HCl (50 mL) was added. The aqueous solution was extracted with ethyl acetate. The combined organic extracts were dried ($MgSO_4$) and evaporated to give an oil. The crude oil was purified by column chromatography [eluant: ethyl acetate/hexane (7:3)]. Yield: 1.4 g (84%)
^1H δ($CDCl_3$) : 0.87 (m, 15H), 1.25 (br, 24H), 2.65 (m, 8H), 3.67 (m, 12H), 4.12 (t, 2H), 4.27 (t, 4H).

Compound (5)

Compound **(4)** (1.4 g, 2.0 mmol) and tetraethylene glycol (2.0 g, 5 equiv.) were dissolved in CH_2Cl_2 (30 mL). DMAP (30 mg, 0.1 equiv.) and DCC (0.9 g, 2 equiv.) were added and the mixture was stirred at room temperature overnight. The solution was filtered and then reduced under pressure. Water was added to the residue and extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$) and evaporated to yield an oil. The

crude oil was purified by column chromatography [eluant: methanol/dichloromethane (3:97)]. Yield: 1.1 g (62%)
^{1}H δ($CDCl_3$) : 0.87 (m, 15H), 1.25 (br, 24H), 2.62 (m, 8H), 3.65 (m, 26H), 4.12 (t, 2H), 4.26 (m, 6H).

Compound (B)

Compound (5) (0.91 g, 1.1 mmol) and undecanoic acid disulphide (D) (0.23 g, 0.53 mmol) were dissolved in CH_2Cl_2 (30 mL). DMAP (13 mg, 0.1 equiv.) and DCC (0.24 g, 1.1 equiv.) were added and the mixture was stirred at room temperature overnight. The solution was filtered and then reduced under pressure. The crude oil was purified by column chromatography [eluant: hexane/ethyl acetate (1:9)]. Yield: 0.25 g (18%)
^{1}H δ($CDCl_3$) : 0.87 (m, 15H), 1.25 (br, 56H), 2.28 (t, 2H), 2.32 (t, 2H), 2.65 (m, 8H), 2.68 (t, 4H), 3.65 (m, 24H), 4.10 (m, 2H), 4.23 (m, 8H).

Compound (A)

Further elution [hexane/ethyl acetate (1:9)] from the column of (B) gave symmetrical disulphide (A) as the second product. Yield: 0.42 g (26%)
^{1}H δ($CDCl_3$) : 0.87 (m, 30H), 1.25 (br, 80H), 2.32 (t, 4H), 2.65 (m, 16H), 2.68 (t, 4H), 3.65 (m, 48H), 4.11 (t, 4H), 4.25 (m, 16H).

Compound (C)

Compound (A) (0.1 g, 0.048 mmol) was dissolved in ethanol/water mixture (20:3) (23mL). Triphenyl phosphine (0.03 g, 2.9 equiv.) was added and the mixture was stirred at room temperature for 3 days. The solvent was evaporated under reduced pressure and the residue was taken up in CH_2Cl_2. The organic solution was dried ($MgSO_4$) and evaporated. The crude product was purified by column chromatography [eluant: hexane/ethyl acetate (1:9)]. Yield: 49 mg (49%)
^{1}H δ($CDCl_3$) : 0.87 (m, 15H), 1.25 (br, 24H), 2.32 (t, 2H), 2.52 (m, 2H), 2.68 (t, 8H), 3.65 (m, 24H), 4.12 (t, 2H), 4.25 (m, 8H).

16-Iodohexadecanoic acid (6)

16-Hexadecanolide (5.0 g, 0.02 mol) was added to a mixture of HI (30 g) and acetic acid (20 g). The mixture was heated to 100 °C overnight. After cooling it was poured into a cold sodium thiosulphate solution (150 mL, 10%) and extracted using CH_2Cl_2. The combined organic extracts were dried (MgSO4) and evaporated to give a white solid. The crude product was recrystallised from diethyl ether. Yield: 4.8 g (64%)
^1H δ($CDCl_3$) : 1.28 (br, 22H), 1.65 (br, 2H), 1.85 (m, 2H), 2.35 (t, 2H), 3.18 (t, 2H).

16-Mercaptohexadecanoic acid (N)

A mixture of 16-iodohexadecanoic acid **(6)** (4.0 g, 10.4 mmol) and thiourea (0.88 g, 1.1 equiv.) in ethanol (100 mL) was refluxed overnight under N_2 atmosphere. The solution was cooled to room temperature. Sodium hydroxide (1.0 g in 10 mL of water) wad added and the mixture was further heated for 2 hr. After cooling to room temperature 1N HCl was added and the product was extracted using CH_2Cl_2. The organic extract was dried and evaporated to give a white solid. Yield: 2.1 g (69%)
^1H δ($CDCl_3$) : 1.28 (br, 22H), 1.62 (br, 4H), 2.35 (t, 2H), 2.50 (t, 2H).

Hexadecanoic acid disulphide (M)

Method 1. 16-Mercaptohexadecanoic acid **(N)** (1.5 g, 5.2 mmol) and triethylamine (1.5 mL, 2.1 equiv.) were dissolved in CH_2Cl_2/CH_3OH(1:1) mixture (20 mL) and cooled. A solution of methanol and iodine was added until excess I_2 was present. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2. The solution was acidified using 3N HCl and little methanol was added to dissolve insoluble solid. The organic phase was separated, dried ($MgSO_4$) and evaporated to yield a solid residue. The product was recrystallised from acetone. Yield: 0.32 g (21%)

Method 2. A mixture of 16-iodohexadecanoic acid **(6)** (4.0 g, 10.4 mmol) and thiourea (0.88 g, 1.1 equiv.) in ethanol (100 mL) was refluxed overnight at

room atmosphere. The solution was cooled to room temperature. Sodium hydroxide (1.0 g in 10 mL of water) wad added and the mixture was further heated for 2 hr. After cooling to room temperature 1N HCl was added and the insoluble product was collected by filtration. Yield: 2.2 g (73%)

^{1}H δ($CDCl_3$) : 1.28 (br, 44H), 1.62 (br, 8H), 2.36 (t, 4H), 2.68 (t, 4H).

Compound (L) (Figure 3)

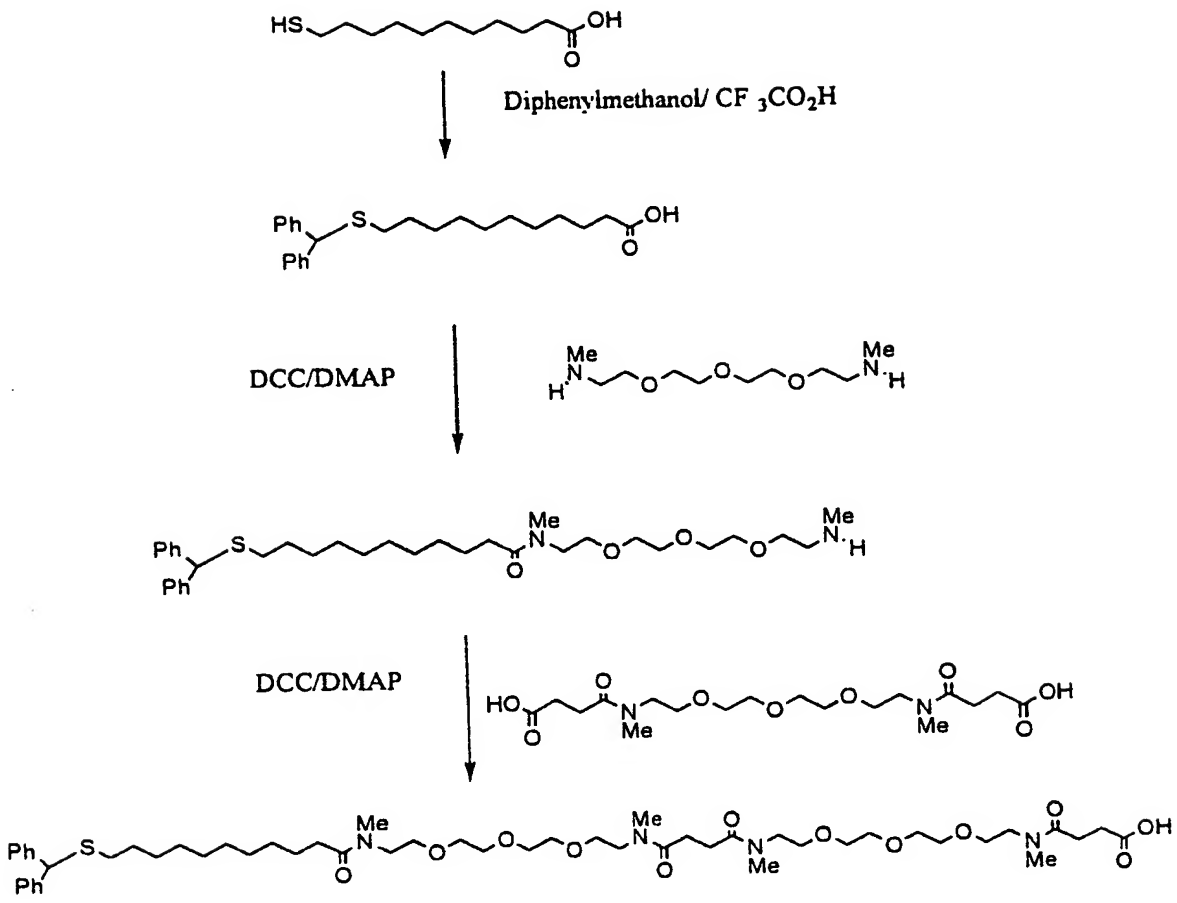
Compound (5) (0.20 g, 0.23 mmol) and hexadecanoic acid disulphide (M) (0.12 g, 0.21 mmol) were dissolved in CH_2Cl_2 (20 mL). DMAP (5 mg, 0.1 equiv.) and DCC (53 mg, 1.1 equiv.) were added and the mixture was stirred at room temperature for 48 hr. The solution was filtered and then reduced under pressure. The crude oil was purified by column chromatography [eluant: hexane/ethyl acetate (15:85)]. Yield: 31 mg (10%)
^{1}H δ($CDCl_3$) : 0.85 (m, 15H), 1.22 (br, 76H), 2.32 (t, 4H), 2.65 (m, 8H), 2.70 (t, 4H), 3.65 (m, 24H), 4.12 (m, 2H), 4.25 (m, 8H).

Compound (K)

Further elution [hexane/ethyl acetate (1:9)] from the column of (L) gave symmetrical disulphide (K) as the second product. Yield: 0.42 g (26%)

16-Mercaptohexadecanol (O)

16-mercaptohexadecanoic acid (N) (0.2 g, 0.7 mmol) was dissolved in dry THF (20 mL) and borane dimethyl sulphide (1 mL, 1.1 equiv.) was slowly added under N_2 atmosphere. The mixture was stirred at room temperature for 4 hr. Water was added to the solution was extracted using ethyl acetate. The organic extract was washed with 1N HCl, water and saturated sodium bicarbonate solution successively. After drying ($MgSO_4$), the solvent was evaporated to give a white powder. Yield: 87 mg (46%)
^{1}H δ($CDCl_3$) : 1.28 (br, 24H), 1.58 (br, 8H), 2.52 (q, 2H), 3.63 (t, 2H).



11-(Diphenylmethylthio)-undecanoic Acid

A solution of 11-mercaptoundecanoic acid (300 mg, 1.3mmol) and diphenylmethanol (255 mg, 1.3 mmol) in trifluoroacetic acid was stirred at RT, under nitrogen for 30 min. Trifluoroacetic acid was evaporated under high vacuum, the residue dissolved in ether (20 ml), washed with water (3X 20 ml), dried and evaporated. The residue was purified by chromatography (light pet. - ethyl acetate, 80: 20- 50:50) on silica to give the desired product (220 mg, 42%), m.p. (Found C, 74.55; H, 8.77. $C_{24}H_{34}O_2S$ requires C, 74.96; H, 8.39) ms (EI) 384.

^1H nmr ($CDCl_3$)1.22-1.29 (m, 12H, CH_2), 1.58 (m, 4H, CH_2), 2.34 (t, 2H, CH_2-CO_2H), 2.37 (t, 2H, CH_2-S), 5.13 (s, 1H, CH-Ph), 7.20-7.44 (m,10H, aromatic H).

(N-Methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide

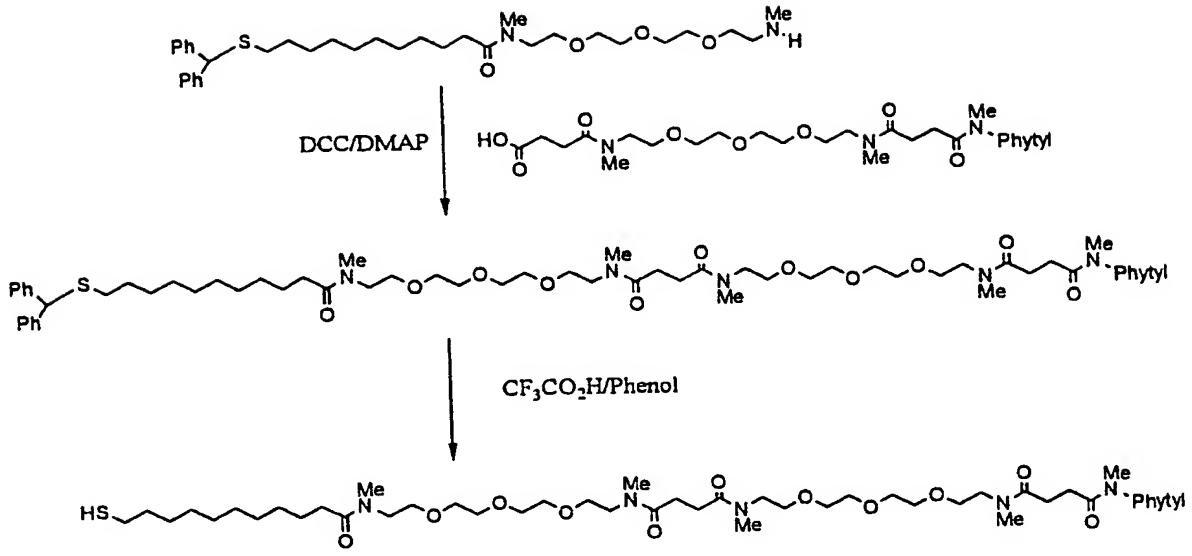
To a solution of diphenylundecanoic amine (700 mg, 1.82mmol) and bisdimethylamino-tetraethyleneglycol diamine (2.0g, 9.1mmol) in dichloromethane (7 ml), DCC (565 mg, 2.74mmol) and DMAP (75 mg, 0.62mmol) dissolved in dichloromethane (3 ml) was added. The mixture stirred at RT under nitrogen for 48h. The solvent evaporated and the residue was purified by chromatography (dichloromethane: MeOH 90: 10 to remove urea salt and dichloromethane: MeOH: NH_3 80: 20: 2) to give the product (390 mg, 37%) as a colourless liquid. (Found C, H, N $C_{34}H_{54}N_2O_4S$ requires C, 69.58; H, 9.27; N, 4.77) ms (EI) 587.

^{1}H nmr ($CDCl_3$)1.22 (m, 12H, CH_2), 1.58 (m, 4H, CH_2), 2.38 (t, 2H, CH_2-CO_2H), 2.49 (t, 2H, CH_2-S), 2.50 (s, 3H, NCH_3-CH_2), 2.83 (t, 2H, CH_2-NCH_3), 2.93 ((s) 3.04 (s), 3H, NCH_3-CO), 3.54-3.64 (m, 14H, CH_2-O), 5.13 (s, 1H, CH-Ph), 7.20-7.43 (m,10H, aromatic H).

{(N-Methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) hemisuccinamide

To a solution of (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (200 mg, 0.34mmol) and bis-(N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) hemisuccinamide (572 mg, 1.36mmol) in dichloromethane (10 ml), DCC (110 mg, 0.53mmol) and DMAP (13 mg, 0.10mmol) dissolved in dichloromethane (4 ml) was added. The mixture stirred at RT under nitrogen for 24h. The white ppt. Of the urea was filtered and washed with dichloromethane. More dichloromethane (50 ml) was added and washed with water (2X30 ml), brine (30 ml), dried and evaporated. The residue was then purified by hplc(4% MeOH, 0.5% acetic acid in dichloromethane) on a semi prep column with retention time (29 min) to give the product (160 mg,47%) a colourless liquid. (Found C, H, N $C_{52}H_{84}N_4O_{12}S$ requires C,; H,; N,) ms (ESI) 989 and M+Na 1012.

^{1}H nmr ($CDCl_3$)) 1.25 (m, 12H, CH_2), 1.57 (m, 4H, CH_2), 2.33 (m, 2H, CH_2-CO_2H), 2.37 (m, 2H, CH_2-S), 2.61-2-67 (m, 8H, CO-CH_2-CH_2-CO), 2.95, 2.97,3.06, 3.09, 3.11 ((s) 15H, NCH_3-CO), 3.55-3.61 (m, 32H, CH_2-O), 5.14 (s, 1H, CH-Ph), 7.16-7.41 (m,10H, aromatic H).



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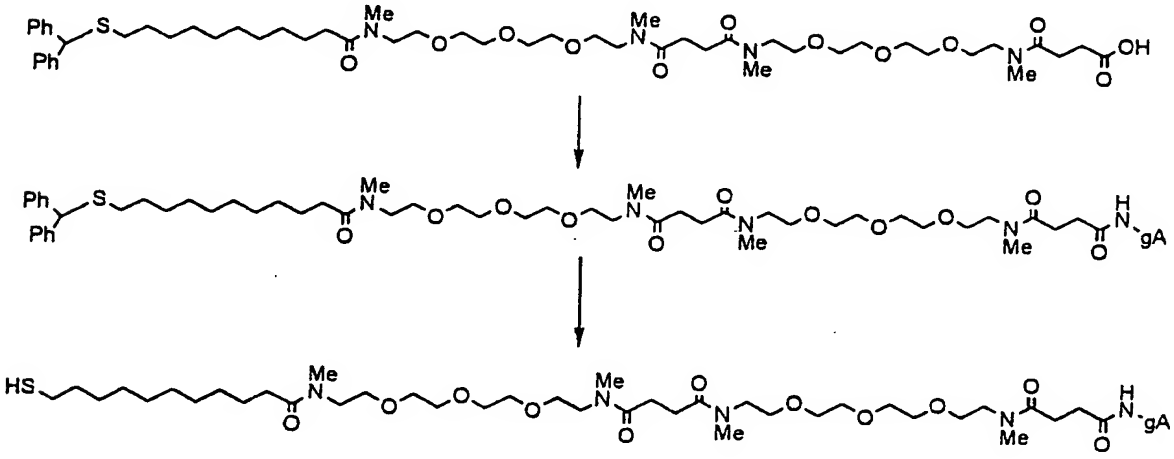
{{(N-Methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) N-methylphytanamine) succinamide

10 To a solution of (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (100 mg, 0.17mmol) and (N-methylphytanamine (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) hemisuccinamide (160 mg, 0.22mmol) in dichloromethane (10 ml), DCC (52 mg, 0.25mmol) and DMAP (6 mg,
15 0.05mmol) dissolved in dichloromethane (3 ml) was added. The mixture stirred at RT under nitrogen for 24h. The solvent evaporated and the residue was purified by chromatography (DCM: MeOH 95: 5) to give the product (180 mg, 83%) as a colourless liquid. (Found C, H, N $C_{73}H_{127}N_5O_{11}S$ requires C,; H,; N,) ms (ESI) 1282 and M+Na 1305.

20 ^{1}H nmr ($CDCl_3$)).82-1.25 (m, 55H, CH_2), 1.59 (m, 4H, CH_2), 2.33 (t, 2H, CH_2-CO_2H), 2.37 (t, 2H, CH_2-S), 2.65-2-68 (m, 8H, CO-CH_2-CH_2-CO), 2.89, 2.95, 2.99, 3.04, 3.09 ((s) 15H, NCH_3-CO), 3.54-3.64 (m, 34H, CH_2-O), 5.13 (s, 1H, CH-Ph), 7.20-7.43 (m,10H, aromatic H).

[([(N-Methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-thioundecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) N-methylphytanamine) succinamide

A solution of [([(N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) N-methylphytanamine) succinamide (100 mg, 0.78mmol) and phenol (144 mg) in trifluoroacetic acid (1 ml), was stirred at RT under nitrogen. After 6h (the hplc indicated the disappearance of the starting material) the trifluoroacetic acid was removed under high vacuum and the residue was purified by hplc (dichloromethane: methanol 93:7) to give a single peak of the product (39 mg, 45%) a colourless liquid. (Found C, H, N $C_{60}H_{117}N_5O_{11}S$ requires C,; H,; N,) ms (CI) 1117 (M+1) ^{1}H nmr ($CDCl_3$) 82-1.25 (m, 55H, CH_2), 1.59 (m, 4H, CH_2), 2.35 (m, 2H, CH_2-CO_2H), 2.60 (m, 2H, CH_2-S), 2.65-2-68 (m, 8H, CO-CH_2-CH_2-CO), 2.89, 2.95, 2.99, 3.04, 3.09 ((s) 15H, NCH_3-CO), 3.54-3.64 (m, 34H, CH_2-O).

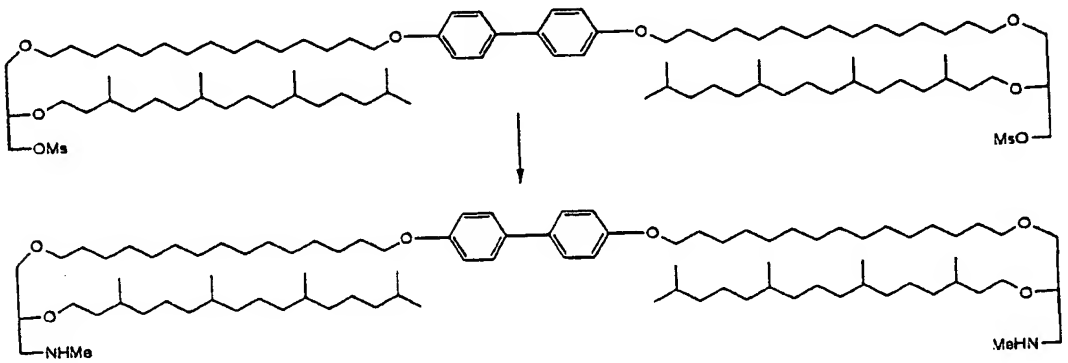


{{(N-Methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) gramicidin-lysine) succinamide

To a solution of ((N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-(diphenylmethylthio)-undecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) hemisuccinamide (40 mg, 0.04mmol) and gramicidin lysine (30 mg, 0.02mmol) in dichloromethane (3 ml), DCC (10.5 mg, 0.05mmol) and DMAP (6 mg, 0.05mmol) dissolved in dichloromethane (1 ml) was added. Pyridine (0.5 ml) was added to dissolve the suspension. The mixture stirred at RT under nitrogen for 72h. The solvent evaporated and the residue purified by reverse phase hplc gradients (A: water B: methanol 50 to 70 to 100 over 60 min) to give the product (8 mg,17%) as fraction 4 with retention time 52 min as white precipitate. ms (ESI) M+Na 2961.

{{(N-Methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane)-11-thio-undecanamide (N-methyl-N'-methyl-3,6,9-trioxa-1,11-diaminoundecane) succinamide) gramicidin-lysine) succinamide

A solution of diphenyl undecanoicTEG-Succ-TEG-Succ-gALys (9 mg, 0.003mmol) and phenol (60 mg) in trifluoroacetic acid (0.4 ml), was stirred at RT under nitrogen. After 3h (the hplc indicated the disappearance of the starting material) the trifluoroacetic acid was removed under high vacuum and the residue was purified by hplc (gradients (A: water B: methanol 50 to 70 to 100 over 30 min) to give the product (1.4 mg, 18%) with retention time 27.53 min as white precipitate. ms (ESI) M+Na 2795.



Bis-dimethylamino membrane spanning lipid [MSL dimethyldiamine]
A solution of the C_{38}-dimesylate (750mg, 0.5mmol) was added to anhydrous methylamine (19mL) at -12°C. The reaction mixture, in a pressure tube, was allowed to warm to room temperature and was gently stirred for 48 h. The excess methylamine was removed and the residue was stirred with excess anhydrous potassium carbonate in dichloromethane for 3h. The mixture was filtered through barium carbonate and the solvent removed to give a waxy solid. Chromatography on silica gel with 1% ammonia and 4% methanol in dichloromethane followed by 1% ammonia and 8% methanol in dichloromethane as eluent gave the C_{38}-diamine (423mg, 62%) as a white waxy solid.
^{1}H nmr ($CDCl_3$) 0.80-1.89 (130H, m, aliphatics), 2.49 (6H, s, NMe), 2.76 (4H, m, CH_2N), 3.36-3.78 (14H, m, OCH and OCH_2), 3.98 (4H, t, ^3J 6.5 Hz, CH_2OAr), 6.95 and 7.46 (8H, AA'BB', biphenyl). $^m/_e$ 687.5 (M^{2+}) (M^+ requires 1374.3).

Example 1 Bilayer: comparison of bottom layer

A freshly prepared evaporated gold thin film (1000A) on glass with chromium under-layer (50 A) was clamped in a containment vessel. Ten microlitres of ethanol solution of compound DLP 1 mM was added into the vessel thus covering the gold electrode. After one hour the electrode was washed with ethanol and dried using nitrogen gas. Five microlitres of PC lipid mixture in ethanol with ionophore valinomycin (200:1)was added to the electrode. The electrode was rinsed then three times with 0.5 mL of 0.1M saline solution. Impedance spectra were obtained before and after 0.1M potassium challenge.

Another freshly prepared evaporated gold thin film (1000A) on glass with chromium under-layer (50 A) was clamped in a containment vessel. Ten microlitres of ethanol solution of compound B 1 mM was added into the vessel thus covering the gold electrode. After one hour the electrode was washed with ethanol and dried using nitrogen gas. Five microlitres of PC lipid mixture in ethanol with ionophore valynomicin (200:1)was added to the electrode. The electrode was rinsed then three times with 0.5 mL of

0.1M saline solution. Impedance spectra were obtained before and after 0.1M potassium challenge.

Yet another freshly prepared evaporated gold thin film (1000A) on glass with chromium under-layer (50 A) was clamped in a containment vessel. Ten microlitres of ethanol solution of compound L 1 mM was added into the vessel thus covering the gold electrode. After one hour the electrode was washed with ethanol and dried using nitrogen gas. Five microlitres of PC lipid mixture in ethanol with ionophore valynomicin (200:1)was added to the electrode. The electrode was rinsed then three times with 0.5 mL of 0.1M saline solution. Impedance spectra were obtained before and after 0.1M potassium challenge.

Impedance at 1kHz, 1 Hz, phase shift minimum and frequency at phase shift minimum for DLP, DLP-C11 and DLP-C16 based bilayers: after KCl challenge are shown in the Table below.

	|Z|, kOhm		|Z| , kOhm		min psi		min frequency
	1 kHz		1 Hz		deg		Hz
DLP	**1.86**	3.5%	**158**	12 %	**39**	4 %	**10-20**
DLP-C11	**1.94**	4 %	**338**	7 %	**50**	3 %	**10**
DLP-C16	**2.34**	4 %	**497**	22 %	**57**	6 %	**10**

Impedances at 1 kHz and 1 Hz and phase shift minimum increase when the monolayer thickness increases from BnDS to C11 to C16 molecules. The frequency minimum on the other hand is only weakly affected by the change.

Example 2 Stability: monolayers

2.1 Stability of C11 and C16 DLP and DLP in H_2O at 50 °C using X-ray Photoelectron Spectroscopy (XPS)

Method:

A freshly prepared evaporated gold thin film (1000A) on glass with chromium under-layer (50 A) was immersed into a vessel containing ethanolic solution 1mM of compound the vessel thus covering the gold electrode. After one hour the electrode was washed with ethanol and dried using nitrogen gas and measured with XPS. The samples were immersed in deionised H_2O for 1hr at 50° C and measured again by XPS. The regions of interest were C1s, O1s and Au 4f. The values quoted below are the average of two measurements on one sample.

Table 1: Carbon and Oxygen, % *(Integrity of monolayer)*

Monolayer	C% before	C% after	O% before	O% after	ratio(C/O) before	ratio(C/O) after
C16-S-S-C16-DLP	78.4	78.9	21.6	21.1	3.63	3.73
C11-S-S-C11-DLP	77.7	77.7	22.3	22.3	3.48	3.48
DLP (standard)	75	76	25	24	3	3.16

Table 2: Carbon and Au % *(surface coverage)*

Monolayer	C (area) before	C (area) after	Au (area) before	Au (area) after	Au/C ratio before	Au/C ratio after
C16-S-S-C16-DLP	5377	6012	20848	22826	3.88	3.80
C11-S-S-C11-DLP	5511	5637	24114	26751	4.38	4.74
DLP (standard)	5454	5319	47854	56988	8.77	10.7

Table 3: O% : Au % *(surface coverage)*

Monolayer	O (area) before	after	Au (area) before	after	Au/O ratio before	after
C16-S-S-C16-DLP	4015	4073	20848	22826	5.20	5.59
C11-S-S-C11-DLP	4012	4103	24114	26751	6.0	6.52
DLP (standard)	4635	4066	47854	56988	10.3	14.0

Table 1 shows that the ratio between the carbon and oxygen content of various DLP's monolayers. The variation is within the experimental error indicating that the molecules did not disintegrate and if any desorption occurred it would be take place in the form of disulfide bond breaking from the Au surface.

Table 2 . shows a comparison of carbon and Au content of various DLP's monolayers before and after 50°C ethanol immersion. The result show that C16-DLP is the least effected by immersion of H_2O at 50 °C, C11-DLP is effected slightly and the greatest effect seen with DLP.

Table 3. shows a comparison of oxygen content of various DLP's monolayers before and after 50°C ethanol immersion. The result show that C16-DLP is the least effected by immersion of H_2O at 50 °C, C11-DLP is effected slightly and the greatest effect seen with DLP. This suggests that the further the monolayer (i.e. the longer molecule) is from the gold surface the more protected it remains.

2.2 Stability of C11 and C16 DLP and DLP in Ethanol at 50 ° C using XPS

Method:

Au thin film were sputtered onto Si substrate with Ti interlayer. The samples were immersed for 1hr at room temperature and measured with XPS. Then the samples were immersed in ethanol for 1hr at 50° C and measured again by XPS. The regions of interest were C1s, O1s and Au 4f. The values quoted below are the average of two measurements on one sample

Table 4: Monolayer composition and Au percentage detected.

Monolayer	C% before	after	O% before	after	Au% before	after
C16-S-S-C16-DLP	67.7	67.4	22.2	22.0	10.1	10.6
C11-S-S-C11-DLP	64.2	64.4	20.5	19.1	15.3	16.5
DLP (standard)	65.0	63	20.0	18.9	15.0	18.1
C18-thiol	54.4	51.3	-	-	45.6	48.7

Table 4 shows a trend where the sample immersed in ethanol at 50° C the Au percentage increased indicating a desorption of the outer layer. Also, this effect appeared to be dependent on the length of the molecules, the longer the molecules the less the increase in Au percentage is observed.

Table 5: Carbon and Oxygen percentage of DLP's monolayers

Monolayer	C% before/	after	O% before /	after	ratio before	/after
C16-S-S-C16-DLP	77.8	78	22.2	22.0	3.5	3.55
C11-S-S-C11-DLP	75.8	77.1	24.2	22.9	3.13	3.37
DLP (standard)	76.5	76.9	23.5	23.1	3.26	3.33

The ratio of carbon to oxygen is a measure of monolayer integrity. The ratio for each of the monolayer do not changes significantly suggesting that the molecules do not disintegrate.

Table 6 Carbon and gold of DLP's monolayers *(surface coverage)*

Monolayer	C (area) before	after	Au (area) before	after	Au/C ratio before	after
C16-S-S-C16-DLP	5948	5807	22849	23385	3.84	4.02
C11-S-S-C11-DLP	5216	5163	24641	28138	4.72	5.5
DLP (standard)	1766	812	8176	4620	4.63	5.69
c18-thiol	950	534	16017	10473	16.9	19.6

Table 7 Oxygen and gold of DLP's monolayers *(surface coverage)*

Monolayer	O (intensity) before	after	Au (intensity) before	after	Au/O ratio before	after
C16-S-S-C16-DLP	4331	4165	22849	23385	5.28	5.61
C11-S-S-C11-DLP	4212	4115	24641	28138	5.85	6.84
DLP (standard)	1384	755	8176	4620	5.91	6.2

Example 3 Bilayer: transient

A freshly prepared evaporated gold thin film (1000A) on glass with chromium under-layer (50 A) was clamped in a containment vessel. Ten microlitres of ethanol solution of compound DLP 1 mM was added into the vessel thus covering the gold electrode. After one hour the electrode was washed with ethanol and dried using nitrogen gas.

Five microlitres of PC lipid mixture in ethanol with ionophore valynomicin (200:1)was added to the electrode. The electrode was rinsed then three times with 0.5 mL of 0.1M saline solution. A transient technique was used to measure sensor response.

A train of potential pulses was applied to the electrode using a commercial potentiostat, EG&G 263M Princeton with AMLAB computer control data acquisition system (pulse duration 50 msec, delay between pulses 950 msec). The currect response of the system was measured before and after 0.1M potassium challenge. The shape of the current response changes upon changes in the concentration of potassium ions.
The current can be fitted using two exponential function with time constants tau1 and tau2. The electrolyte concentration can be correlated with one of the time constant that is related with the membrane conductance.

Example 4 Ferritin gating experiments.

Aim:

To demonstrate that a ferritin gating response can be achieved with membrane systems incorporating C11-amide gAYY.

Method:

1st layer solutions:

i) AM300

DLP	10mM	1500µL
MSLOH	1mM	225µL
MAAD	10mM	750µL
MSL4XB	0.1mM	11.25µL
gAYY	0.01mg/mL	579µL

+ 46.9mL EtOH

ii) 2,000:1, C11-DLP/C11-amide gAYY

DLP-C11	10mM	25µL
$HS(CH_2)_{10}CO_2H$	1mM	125µL
C11-amide gAYY	**3.61µM**	**34.7µL**

+ 815.3µL EtOH

iii) 5,000:1, C11-DLP/C11-amide gAYY;

DLP-C11	10mM	25µL
$HS(CH_2)_{10}CO_2H$	1mM	125µL
C11-amide gAYY	**3.61µM**	**138.6µL**

+ 711.4µL EtOH

Standard, hand assembled blocks were used.

1st layer formation:

Fresh slides were obtained from the evaporator and used immediately. Slides were assembled in blocks and to each cell 40µL of 1st layer solution added.

Blocks were incubated with 1st layer solution for ~ 2 - 3 hours at room temperature, whereupon excess solution was removed, and each cell rinsed thoroughly with ethanol from a wash bottle. Residual ethanol was removed by vigorous shaking and blocks inverted and air-dried.

2nd layer formation:

5×10^4 / 4 gA-5XB conducting membranes were assembled using 10 mM DPEPC:GDPE 7:3.After assembly membranes were challenged with;

i) **5µL, 0.1mg/mL Streptavidin, PBS wash(3x)**

ii) **5µL, 0.05mg/mL anti-Ferritin Fab', PBS wash (3x)**

ii) **100µL, 100pM Ferritin**

N.B.: 2 different substrates were used; (i.e. deposition process - evaporation)

i) polycarbonate / Cr + Au

ii) glass / Cr + Au

Results:

Table 1: Comparison of Ferritin Gating responses (50pM final conc.)

1st layer	No. Cells	1 Hz Intercept (kHz)		1 kHz Intercept (kHz)		Freq. Min. Phase (Hz)		NMS (-/ks)		Tau (sec.)		% Gating	
		Av.	C.V.	Av.	C.V.	Av.	C.V.	Av.	C.V.	Av.	C.V.	Av.	C.V.
AM300	4	**140**	*3.6*	**1.8**	*4.8*	**18.**	*20.1*	**1.231**	*9.9*	**528**	*14.7*	**52**	*3.9*
2,000:1	5	**502**	*10.7*	**2**	*11*	**413**	*100.6*	**0.651**	*22.4*	**795**	*7.6*	**51**	*12.4*
5,000:1	6	**416**	*5*	**2**	*5.6*	**37**	*33*	**0.669**	*24.1*	**785**	*26*	**42**	*9.5*
AM300	7	**118**	*23.8*	**1.4**	*2.7*	**43.7**	*10.1*	**1.671**	*7.7*	**247**	*10.4*	**43**	*9.5*
2,000:1	14	**446**	*18.8*	**2.1**	*7.7*	**23.9**	*16.1*	**1.325**	*17.2*	**242**	*13.3*	**36.5**	*14*
5,000:1	6	**453**	*3.4*	**1.7**	*5.2*	**16.7**	*16.3*	**1.287**	*14.8*	**290**	*9.1*	**40**	*8.6*
AM300	3	**149**	*3.9*	**1.8**	*3.9*	**31.8**	*8.1*	**0.976**	*5.8*	**517**	*8*	**49.3**	*3.3*
2,000:1	15	**457**	*2.3*	**1.9**	*3.4*	**42.3**	*12.4*	**0.581**	*7*	**1391**	*16*	**53**	*14.5*

Conclusion:

A ferritin gating response can be elicited from membranes assembled with C11-amide modified gAYY. From the 3 different data sets, the magnitude of the gating response is comparable to the standard AM300 system, however there is some variation in the speed of response and initial membrane conductivity.

Example5 Stability trials testing C11-amide gAYY.

Aim:

To determine whether the inclusion of a C11-amide modified gAYY improves membrane stability upon storage.

Method:

1st layer solutions:

i) "AM300"

DLP	10mM	1500µL
MSLOH	1mM	225µL
MAAD	10mM	750µL
MSL4XB	0.1mM	11.25µL
gAYY	0.01mg/mL	579µL
+ 807.5µL EtOH		

ii) 2,000:1 DLP-C11/C11-ester gAYY;

DLP-C11	10mM	25µL
$HS(CH_2)_{10}CO_2H$	1mM	125µL
C11-ester gAYY	0.01mg/mL	42.3µL
+ 46.9mL EtOH		

iii) 5,000:1, C11DLP/C11-amide gAYY;

DLP-C11	10mM	25µL
$HS(CH_2)_{10}CO_2H$	1mM	125µL
C11-amide gAYY	**3.61µM**	**138.6µL**

+ 711.4µL EtOH

Manual assembled blocks were used.
Substrate: polycarbonate / Cr + Au (Evaporated)

1st layer formation:

Fresh slides were obtained from the evaporator and used immediately. Slides were assembled in blocks and to each cell 40µL of 1st layer solution added.

Blocks were incubated with 1st layer solution for ~ 2 - 3 hours at room temperature, whereupon excess solution was removed, and each cell rinsed thoroughly with ethanol from a wash bottle. Residual ethanol was removed by vigorous shaking and blocks inverted and air-dried.

2nd layer formation:

Sealed, non-conducting membranes were prepared using 10mM DPEPC/GDPE 7:3.

Blocks were stored at room temperature in PBS and the impedance measured at 0.1Hz at periodic intervals.

Results:

Averaged Impedance Plots:

The averaged impedance plots of the test system (i.e. with the inclusion of C11-amide gAYY, 5,000:1) and 2 controls; AM300 and a membrane system with C11-ester analogues only were determined. These membranes were stored in PBS at room temperature, with the first 7 days of data being shown.

The AM300 showed a rapid increase in membrane leakage after 7 days storage. In comparison, when both the C11-SH passivity layer and amide linkages are incorporated into the tethered gramicidin component (i.e. C11-amide gAYY), the membrane remained well sealed, with no change in stability after 7 days storage.

Membranes assembled from C11-ester analogues only showed improvement over AM300 but were not as stable as the C11-amide gAYY. Similar results were obtained after 3 weeks and 5 weeks storage.

Conclusion:

It has been shown that a stable, fully hydrated bilayer membrane can be achieved by improving the chemical stability within the reservoir region. When membranes are assembled with a C11-amide modified tethered gramicidin, membranes are stable for at least 5 weeks at room temperature with no significant loss of sealing ability.

The inclusion of a passivity layer (C11-SH tether) improves the Au-S attachment, while the introduction of amide linkages are more resistant to hydrolysis. These improvements minimise the ability of the tethered gramicidin to detach from the substrate surface, allowing it to transfer between membrane layers.

This result represents the most significant advance as it overcomes the rapid degradation / leakage associated with the short term stability of current membrane based biosensor systems.

The membrane stability with this new architecture may be further enhanced by minimising degradation due to mechanical leakage. The use of a slide laminate will help to define the cell area in a more controlled manner and reduce the likelihood of membrane leakage underneath the teflon inserts of the current block assembly.

It is also important to note that the membrane stability achieved is in the absence of any membrane spanning component in the bilayer. Further improvements in stability would most likely be achieved with the inclusion of a transmembrane lipid.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:-

1. A membrane based biosensor, the biosensor including an electrode, a passivating layer bound to the electrode, a lipid membrane incorporating ionophores, the conductivity of the membrane being dependent on the presence or absence of an analyte, an ionic reservoir between the membrane and the passivating layer, and reservoir spanning molecules spanning the ionic reservoir the molecules being covalently attached at one end to the membrane and at the other to the passivating layer.

2. A membrane based biosensor as claimed in claim 1 in which the biosensor includes a plurality molecules of following general structure:

A-B-C-D

in which:

A is a hydrophobic group of between 2-50 methylene units in length;

B is a group which spans the ionic reservoir;

C is a group capable of hydrogen bonding, forming van der Waal's interactions, ionic bonding or covalent bonding with other molecules within the passivating layer; and

D is a group which binds to the conducting substrate.

3. A membrane based biosensor as claimed in claim 2 in which A is a hydrocarbon group of between 2-50 methylene units long, a phytanyl group, an unsaturated hydrocarbon of between 2-50 methylene groups long, a membrane spanning lipid, an archaebacterial lipid, a lipid hydrocarbon group, or a gramicidin derivative.

4. A membrane based biosensor as claimed in claim 3 in which A is a hydrocarbon group or an unsaturated hydrocarbon group of between 8-26 methylene units long or a gramicidin derivative.

5. A membrane based biosensor as claimed in any one of claims 2 to 4 in which B is an oligoethylene glycol of between 4 to 20 ethylene glycol units long.

6. A membrane based biosensor as claimed in any one of claims 2 to 4 in which B is repeating subunits of oligoethylene glycol of between 2 and 6 ethylene glycol units, the subunits being linked together via ester, amide or other linkages.

7. A membrane based biosensor as claimed in claim 6 in which linkages do not promote hydrogen bonding between the groups spanning the reservoir.

8 A membrane based biosensor as claimed in claim 6 in which the linkages are tertiary amides.

9. A membrane based biosensor as claimed in claim 2 in which C includes a secondary amide capable of hydrogen bonding with other amides; a hydrocarbon group capable of forming van der Waals interactions with other hydrocarbon groups, or a polymerisable group.

10. A membrane based biosensor as claimed in claim 9 in which C is a saturated hydrocarbon group of between 2 to 50 methylene units long, more preferably 8 to 30 methylene units long.

11. A membrane based biosensor as claimed in claim 2 in which D is a thiol, disulfide, sulfide, phosphine, silane or carboxylate.

12. A membrane based biosensor as claimed in any one of claims 1 to 11 in which the biosensor includes a plurality molecules of following general structure:

C-D

in which:

C is a group capable of hydrogen bonding, forming van der Waal's interactions, ionic bonding or covalent bonding with other molecules within the passivating layer; and

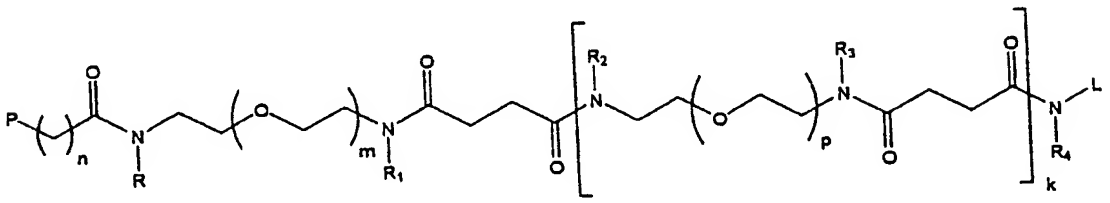
D is a group which binds to the conducting substrate.

13. A membrane based biosensor as claimed in claim 12 in which C includes a secondary amide capable of hydrogen bonding with other amides; a hydrocarbon group capable of forming van der Waals interactions with other hydrocarbon groups, or a polymerisable group.

14. A membrane based biosensor as claimed in claim 11 or claim 12 in which C is a saturated hydrocarbon group of between 2 to 50 methylene units long, more preferably 8 to 30 methylene units long.

15. A membrane based biosensor as claimed in claim 12 in which D is a thiol, disulfide, sulfide, phosphine, silane or carboxylate.

16. A membrane based biosensor as claimed in claim 2 in which the molecule is of the formula:-



where: n = 8 to 16
m = 1 to 10
p = 1 to 10
k = 0 to 10
R, R_1, R_2, R_3, R_4 = independently H, methyl, ethyl
L = hydrocarbon such as a phytanyl chain, or other lipidic hydrocarbon
P = a thiol, disulfide, sulfide, or other group for attaching to metal surfaces such as gold, platinum, palladium, silver etc; or a silane or alkoxy silane or chloro silane for attaching to silica or metal oxide.

17. A membrane based biosensor as claimed in any one of claims 1 to 16 in which the passivating layer has reduced permeability towards water and towards ions thus protecting the surface of the conductive substrate from destabilising effects due to water or ions.

18. A membrane based biosensor as claimed in any one of claims 1 to 17 in which the reservoir spanning molecules are atached to the passivating layer via an ester, ether or amide linkage, preferably by an amide linkage, most preferably a secondary amide.

19. A membrane based biosensor as claimed in any one of claims 1 to 18 in which the reservoir spanning molecules are atached to the lipid membrane

via an ester, ether or amide linkage, preferably by an amide linkage, most preferably a secondary amide.

Figure 1

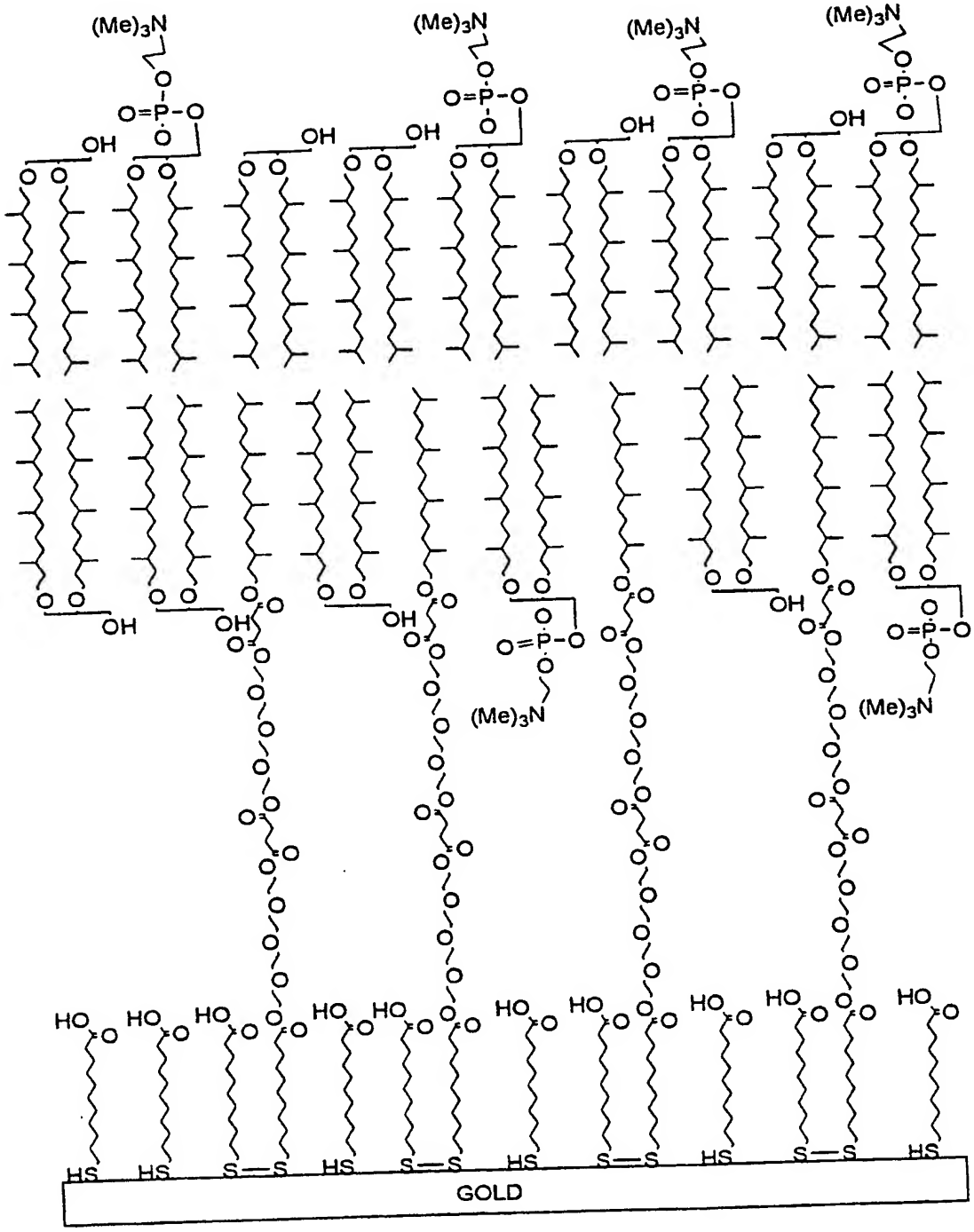


Figure 2

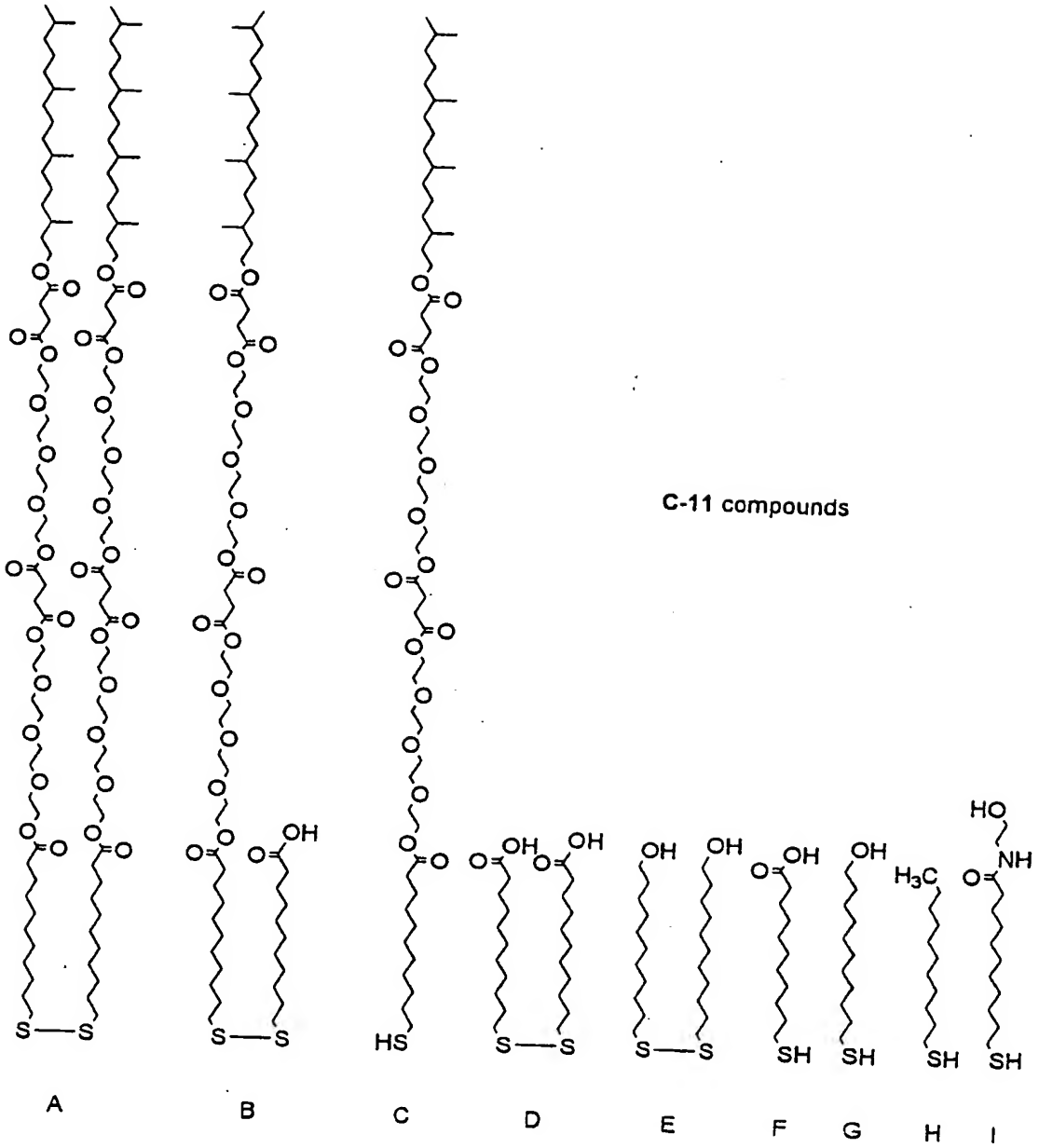
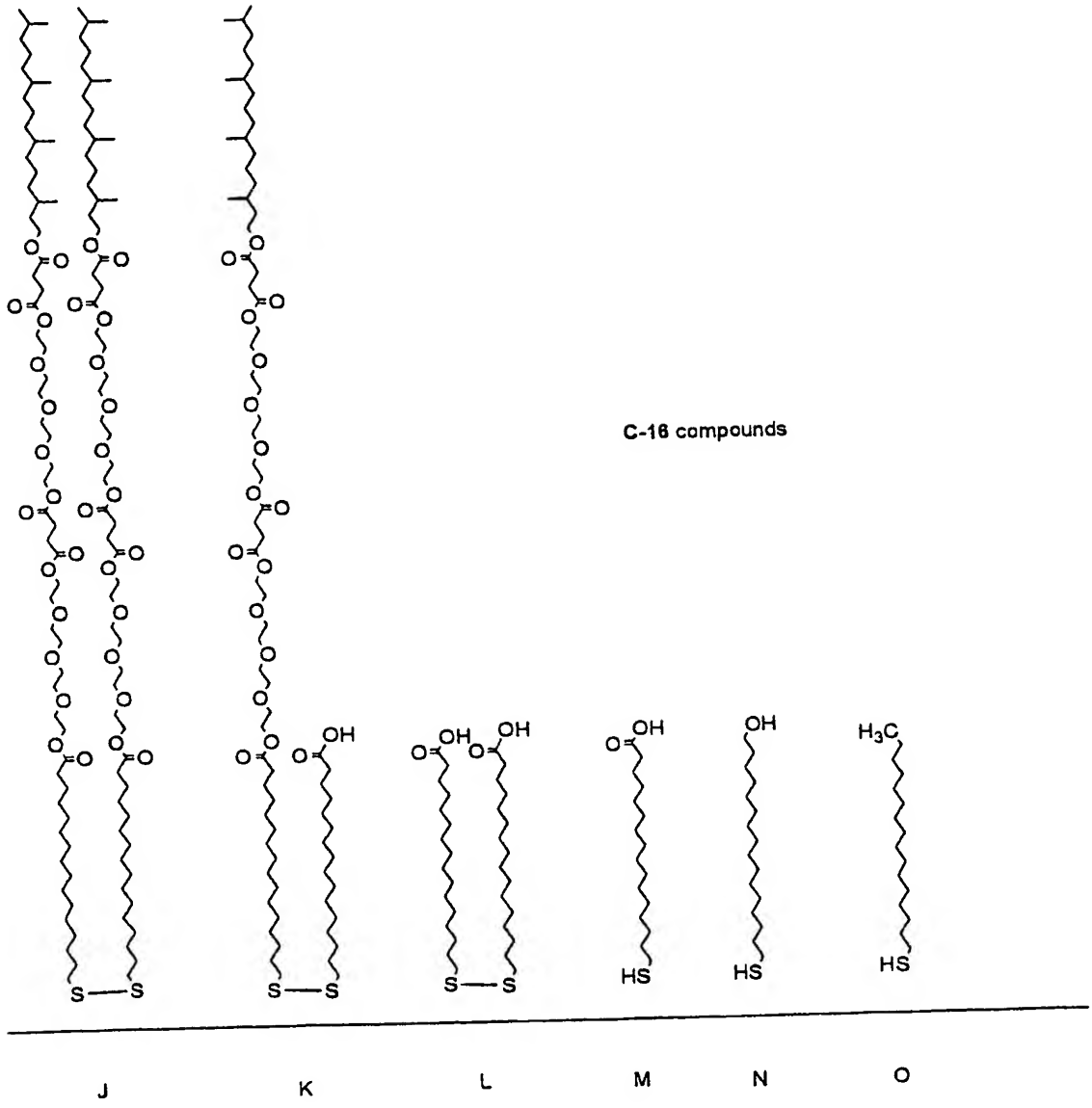


Figure 3



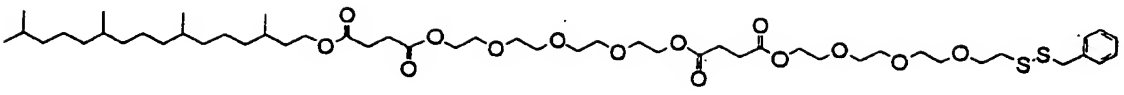
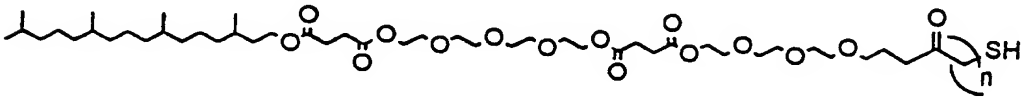


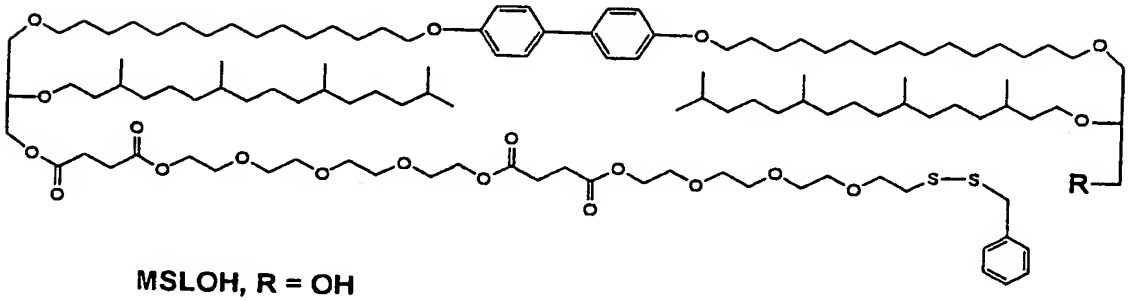
Figure 4



DLP-C11, n = 10

DLP-C16, n = 15

Figure5



MSLOH, R = OH

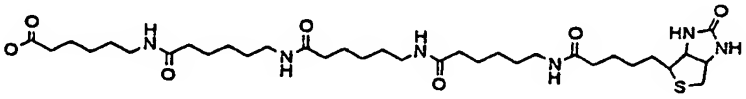
MSL4XB, R = 

Figure 6

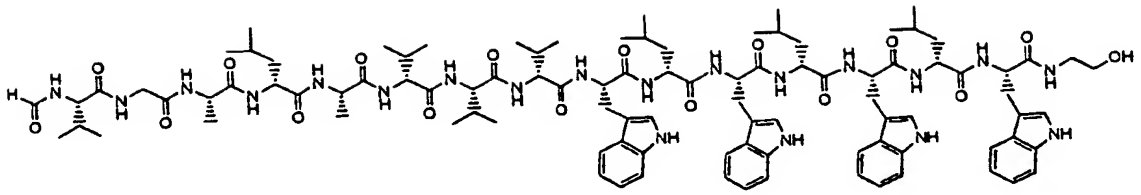


Figure 7

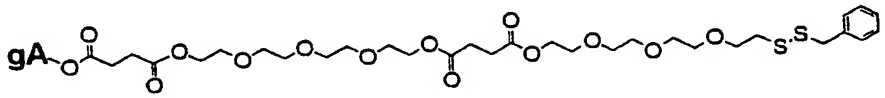


Figure 8

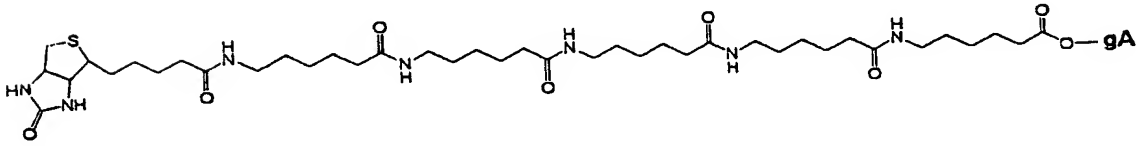


Figure 9

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00423

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 27/327, 27/333

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: G01N 27/327, 27/333

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: membrane and (ion or ionaph:) and passivat:
CAPLUS: (biosensors or bioelectrodes) and (membranes) and (ionophorels)
US Patent Database: biosensor and membrane and passivat

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92/17788 (AUSTRALIAN MEMBRANE AND BIOTECHNOLOGY RESEARCH INSTITUTE 15 October 1992 entire document	1-19
Y	Biosensors and Bioelectronics (1995) vol. 10 Pandey et al. "Tetracyanoquinodimethane mediated glucose sensor based on a self-assembling alkanethiol/phospolipid bilayer" pages 664-674 entire document	1-19

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 1998	-5 AUG 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer STEVEN CHEW Telephone No.: (02) 6283 2248

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00423

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 441 120 A2 (YEDA RESEARCH AND DEVELOPMENT COMPANY, LTD) 14 August 1991 page 3 line 51 - page 4 line 6; figure 2	1-19
Y	WO 97/01092 (AUSTRALIAN MEMBRANE AND BIOTECHNOLOGY RESEARCH INSTITUTE) 9 January 1997 Pages 2-3; figure 4	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 98/00423

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	92/17788	AU	14657/92	CA	2106966	EP	639269
		US	5401378				
EP	441120	AU	69245/91	CA	2033776	IL	93020
		JP	6090736	US	5204239		
WO	97/01092	AU	59926/96	EP	838028		

END OF ANNEX